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Profiling microRNAs in individuals at risk of progression to rheumatoid arthritis

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Abstract

Background: Individuals at risk of rheumatoid arthritis (RA) demonstrate systemic autoimmunity in the form of anti-citrullinated peptide antibodies (ACPA). MicroRNAs (miRNAs) are implicated in established RA. This study aimed to (1) compare miRNA expression between healthy individuals and those at risk of and those that develop RA, (2) evaluate the change in expression of miRNA from "at-risk" to early RA and (3) explore whether these miRNAs could inform a signature predictive of progression from "at-risk" to RA.

Methods: We performed global profiling of 754 miRNAs per patient on a matched serum sample cohort of 12 anti-cyclic citrullinated peptide (CCP) + "at-risk" individuals that progressed to RA. Each individual had a serum sample from baseline and at time of detection of synovitis, forming the matched element. Healthy controls were also studied. miRNAs with a fold difference/fold change of four in expression level met our primary criterion for selection as candidate miRNAs. Validation of the miRNAs of interest was conducted using custom miRNA array cards on matched samples (baseline and follow up) in 24 CCP+ individuals; 12 RA progressors and 12 RA non-progressors.

Results: We report on the first study to use matched serum samples and a comprehensive miRNA array approach to identify in particular, three miRNAs (miR-22, miR-486-3p, and miR-382) associated with progression from systemic autoimmunity to RA inflammation. MiR-22 demonstrated significant fold difference between progressors and non-progressors indicating a potential biomarker role for at-risk individuals.

Conclusions: This first study using a cohort with matched serum samples provides important mechanistic insights in the transition from systemic autoimmunity to inflammatory disease for future investigation, and with further evaluation, might also serve as a predictive biomarker.

Keywords: Rheumatoid arthritis, MicroRNA, At risk, Progression, ACPA, Early RA

Background

Individuals at risk of rheumatoid arthritis (RA) or pre-clinical RA [1, 2] are characterised by the presence of systemic autoimmunity in the form of highly specific anti-citrullinated peptide antibodies (ACPAs) with or without rheumatoid factor (RF) [3]. Increasing research efforts are focusing on tools to identify those at highest risk of progression to RA in whom immunomodulatory therapy could be used as a preventative strategy [4]. We have previously reported on an at-risk cohort defined by the presence of anti-cyclic citrullinated peptide (CCP) antibody and non-specific musculoskeletal (MSK) symptoms [5] in whom progression to inflammatory arthritis (IA) occurred in 50%, after a median of 7.9 months (range 0.1–52.4), 34% within 12 months.

MicroRNAs (miRNAs) are a highly conserved class of short non-coding RNAs that serve as transcriptional

additional miRNAs might be relevant in driving a state of autoimmunity to disease i.e. at the time of disease initiation. No miRNA study to date has focused on individuals at risk of RA.

We took a unique approach of using samples from individuals identified as being at risk and a further sample at the time of their development to RA; first, to compare miRNA expression between health and individuals at risk of RA, and those that develop RA; second, to evaluate for any change in expression of the identified miRNAs with progression along the RA continuum and third, to explore whether these miRNAs could inform a signature predictive of progression from systemic autoimmunity to RA for future evaluation.

Methods

Study design and participants

Prospective CCP cohort study

Since 2007, patients ≥ 18 years of age presenting to primary care services in Yorkshire (UK) with any new, non-specific, MSK symptom(s), who test positive for anti-CCP (CCP+) have been invited to attend regular assessments at the research clinic at Chapel Allerton Hospital, Leeds (UK), as part of the prospective CCP study. The CCP study is sponsored by the University of Leeds, approved by the appropriate research ethics committee (REC; reference 06/Q1205/169). All patients provided written informed consent for the study.

Patient assessments

All patients recruited to the CCP study are assessed at baseline, 3-monthly intervals for the first year, and then as clinically indicated and/or until they developed inflammatory arthritis (IA); defined by the presence of at least one tender and swollen joint confirmed by a rheumatologist. Rheumatologists, trained in the assessment of IA, carry out the clinical assessments. Blood sampling and power Doppler ultrasound scan of the hands and feet are performed at baseline and then at regular intervals until the development of IA. The ultrasound examination was performed on all patients by a single rheumatologist (JLN) experienced in MSK ultrasound. Patients recruited to the study had scans of the wrists, metacarpophalangeal joints (MCPs), proximal interphalangeal joints (PIPs) and metatarsophalangeal joints (MTPs) bilaterally (as well as any other joints if symptomatic). We and others have previously demonstrated the presence of synovitis on ultrasound [9]. CCP + at-risk individuals with ultrasound-identified synovitis (defined as power Doppler signal) of the aforementioned small joints were excluded, as this was considered to be too close to RA on a pathological level. Selection of individuals for our miRNA profiling study was from this at-risk, CCP+ cohort.

Pilot and validation-phase patient cohorts

From our at-risk cohort, 12 CCP+ patients who progressed to RA (American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2010 criteria), termed very early RA (VERA) were available as per the criteria described earlier and selected. Each patient had a blood sample taken at baseline and at the time of detection of synovitis, forming the matched element of this analysis. Twelve healthy controls (HC) were identified via our "ask a friend" approach, tested for and confirmed to be anti-CCP negative, and subsequently also included.

To validate the findings, a further 12 CCP+ patients who progressed to VERA (progressors) and had a matched blood sample available at detection of synovitis were identified. A comparator group consisting of the available 12 CCP+ individuals who did not progress to VERA (non-progressors) and for whom samples were available, were also selected, with a matched sample used 36 weeks after baseline. The 36-week time point was selected following pilot-phase data, indicating a median time to progression to synovitis of 34.5 weeks, enabling a closely matched sample point between the two groups. A further 12 HC were studied. Thus, the validation phase included an identical group to that in the pilot phase and an additional comparator group to enable us to determine whether the miRNAs identified were unique to the development of VERA. Additional file 1 illustrates patient characteristics for the pilot and validation phases.

Isolation and profiling of serum miRNA

Serum microRNAs were isolated according to the manufacturer's protocol using miRNeasy serum plasma kit (Qiagen, UK). For complementary DNA (cDNA) synthesis, Taqman miRNA reverse transcription kit was used (Life Technologies), 3 μl RNA input isolated from serum with Megaplex primer pools Human set v3.0 A and B (Life Technologies) separately. Pre-amplification reactions were performed following manufacturer's protocol using Taqman pre-amplification mastermix, Taqman array human miRNA A and B (Life Technologies). Undiluted pre-amplification product was prepared in a mastermix with Taqman universal mastermix II no UNG and water and loaded into Taqman Low Density TLDA microRNA cards A v3.0 and B set v2.0 (Life Technologies) on an Applied Biosystems 7900HT fast real-time system. See Additional file 2 for detailed methods.

Quantitative reverse transcription (qRT)-PCR on custom miRNA cards

The validation phase was carried out using quantification of the expression of miRNAs of interest using TLDA custom cards (31 candidate miRNAs), and RNU6B was used

miRNAs network analysis

MetaCore™, an extensively used integrated software suite (Thompson Reuters, New York, NY, USA) used for the functional analysis of high-throughput data including microRNA, and based on MetaBase [10, 11] was used for the network analysis.

Statistical analysis

For both phases, we used a rule of thumb of n = 12 per group for pilot studies [12]. As appropriate for pilot studies, the extent of descriptive differences rather than inferential testing (and use of *p* values) was applied. For between-group comparisons, quantile regression, adjusting for age, was used to obtain adjusted between-group differences in median dCt, which was converted to fold difference (FD) (2^{-ddCt}). For within-patient changes, ddCt was calculated then median ddCt was calculated at the group level and converted to fold change (FC). If FD or FC was < 1, -1/(value) was calculated. Fold differences were calculated as 2-(dCt (progressors)-dCt (non-progressors)). Fold changes were calculated as 2-(dCt (follow-up)-dCt (baseline)). In either case, if the value was < 1, it was transformed to -1/FD (or -1/FC as appropriate). Negative values therefore indicate that expression was lower in progressors compared to non-progressors (negative FD), or lower at follow up compared to baseline (negative FC).

prior to analysis (see Additional file 2). GraphPad Prism 5, R and SPSS v.21 software packages were used.

Results

Patient cohorts - progression from CCP+ status to VERA

CCP+ patients (n = 136) with non-specific MSK symptoms were recruited to the prospective "at-risk" clinic: 57 patients progressed to VERA after a median (range) of 8.6 months (0.1–52.4). Of those 57 patients, 29 had no ultrasound-detectable synovitis (including in symptomatic joints) at baseline; of these, 12 available individuals were selected for the pilot phase. A further available 24 patients (12 who progressed to RA and 12 who did not) were selected for the validation phase (Additional file 1).

Pilot phase of serum miRNA profiling

Of the 754 human miRNAs accurately quantified, a number were observed to have different expression profiles between the cohorts. As detailed earlier, the primary criterion for selection of miRNAs of interest was a FD/FC of 4 in expression level (FD/FC ≥4); for within-patient change (CCP+ status to VERA) we also required a pattern

Table 1 List of miRNAs of interest with age-adjusted FD ≥4 between the three studied cohorts in the pilot phase (↑upregulated FC ≥4, ↓downregulated FC ≤ -4)

	HC	CCP	VERA	CCP vs. HC	VERA vs. HC	CCP to VERA (within progressors)		
miR	dCt median (IQR)	dCt median (IQR)	dCt median (IQR)	FD between medians	FD between medians	Median (IQR) ddCt	Median FC	Number upregulated (/12
miR-16	-6.3 (-7.1, -6.0)	-7.1 (-7.6, -7.0)	-7.6 (-8.2, -7.4)	1.7	2.4	-0.4 (-1.1, -0.1)	1.3	10
miR-18a	0.1 (-0.8, 1.3)	1.3 (0.3, 2.0)	-0.1 (-1.0, 0.4)	-2.4	1.1	-1.6 (-2.0, -1.0)	3.1	10
miR-19a	-0.9 (-1.6, 1.0)	-2.4 (-2.8, -1.9)	-3.2 (-3.5, -2.8)	2.9	5.0↑	-0.6 (-1.7, 0.1)	1.5	9
miR-21	-2.8 (-4.1, -2.5)	-3.8 (-4.4, -3.3)	-4.3 (-4.8, -4.1)	2.0	2.7	-0.7 (-1.2, -0.2)	1.6	9
miR-22	4.2 (0.5, 4.8)	3.0 (1.5, 5.1)	0.9 (0.6, 1.5)	2.2	9.4↑	-2.1 (-3.6, -1.5)	4.3↑	**12**
miR-26b	-1.4 (-3.0, 0.2)	-3.1 (-3.4, -1.8)	-3.6 (-4.2, -2.8)	3.3	4.7↑	-0.7 (-2.3, -0.3)	1.7	10
miR-34a	-0.2 (-2.1, 0.8)	-0.1 (-0.3, 1.0)	-0.6 (-2.1, 0.2)	-1.1	1.3	-0.1 (-0.9, 0.3)	1.1	6
miR-101	2.5 (1.7, 3.2)	1.6 (1.3, 1.8)	0.4 (-0.3, 0.9)	1.9	4.3↑	-1.1 (-1.9, -0.6)	2.1	11
miR-132	-1.6 (-2.0, -1.4)	-1.7 (-2.1, -1.6)	-2.5 (-2.7, -2.2)	1.0	1.8	-0.8 (-1.1, -0.3)	1.7	11
miR-142-3p	-2.3 (-4.5, -1.3)	-4.4 (-4.5, -3.8)	-5.0 (-5.2, -4.5)	4.2↑	6.2↑	-0.4 (-1.1, -0.3)	1.4	10
miR-142-5p	3.9 (2.9, 5.7)	3.0 (2.4, 5.5)	1.7 (1.5, 4.3)	1.9	4.7↑	-1.3 (-1.4, -0.3)	2.4	10
miR-146a	-7.3 (-7.5, -6.5)	-7.1 (-7.6, -7.0)	-7.5 (-7.7, -7.3)	-1.2	1.1	-0.5 (-0.8, 0.2)	1.4	8
miR-155	-0.9 (-2.3, 1.5)	0.1 (-0.5, 1.1)	-0.5 (-0.7, -0.3)	-2.1	-1.4	-0.3 (-1.3, 0.1)	1.2	8
miR-195	-2.5 (-3.0, -1.6)	-0.4 (-3.4, 0.2)	-2.9 (-4.2, -0.5)	-4.6↓	1.3	-1.1 (-2.0, -0.5)	2.1	11
miR-197	-3.5 (-3.3, -1.3)	0.6 (-1.5, 2.9)	0.6 (-2.5, 2.2)	-16.8↓	-16.5↓	-0.6 (-2.7, 1.3)	1.5	7
miR-203	2.3 (1.8, 3.0)	3.0 (2.1, 3.0)	2.9 (2.6, 3.4)	-1.5	-1.5	0.0 (-0.4, 0.6)	1.0	6
miR-210	3.9 (0.1, 5.0)	3.1 (2.2, 3.4)	1.5 (1.1, 1.8)	1.8	5.1↑	-1.8 (-2.9, -0.3)	3.4	10
miR-223	-9.3 (-9.6, -8.8)	-9.9 (-9.9, -9.6)	-10.3 (-10.4, -10.0)	1.5	2.0	-0.4 (-0.6, 0.2)	1.3	6/9[a]
miR-361	-0.1 (-1.1, 1.1)	2.1 (-0.5, 2.5)	0.6 (-0.2, 0.7)	-4.7↓	-1.7	-1.5 (-2.1, -1.0)	2.9	11
miR-374[d]	-2.2 (-3.4, -2.1)	-3.2 (-3.7, -2.0)	-4.1 (-4.3, -3.9)	2.0	3.8	-0.6 (-1.0, -0.4)	1.5	12
miR-382	-0.7 (-1.2, 0.5)	1.4 (0.9, 2.7)	-0.1 (-1.2, 0.5)	-4.1↓	-1.5	-2.0 (-2.8, -0.8)	4.1↑	**11**
miR-454[d]	-1.3 (-2.8, 0.2)	-2.3 (-3.2, -0.1)	-3.0 (-3.3, -1.3)	1.9	3.3	-0.5 (-1.1, -0.2)	1.4	10
miR-486-3p	4.3 (2.5, 5.6)	4.9 (2.5, 6.2)	3.5 (3.1, 4.3)	-1.5	1.8	-2.0 (-3.1, 0.2)	**4.1↑**	**9**
miR-520c-3p	2.3 (0.3, 2.9)	-0.4 (-1.6, 2.4)	-1.1 (-1.4, 0.5)	6.3↑	10.6↑	0.3 (-1.7, 0.6)	-1.3	5
miR-579[c]	4.2 (3.5, 5.0)	3.5 (3.1, 3.7)	2.4 (1.8, 2.9)	1.6	3.6	-1.2 (-1.7, -0.3)	2.2	11
miR-590-3P	5.2 (2.9, 5.8)	3.2 (2.3, 3.9)	2.4 (2.2, 3.1)	3.9	6.9↑	-0.9 (-1.1, 0.2)	1.9	8
miR-590-5p	1.2 (0.7, 1.4)	-0.4 (-1.2, -0.2)	-1.3 (-1.6, -0.7)	3.0	5.6↑	-0.5 (-1.1, -0.3)	1.4	10
miR-598	3.7 (3.1, 4.3)	2.8 (2.5, 3.6)	1.5 (1.4, 1.9)	1.8	4.6↑	-1.2 (-1.7, -0.8)	2.2	12
miR-628-5p	3.0 (-8.4, 7.9)	-7.2 (-8.3, 3.9)	-2.9 (-7.8, 2.2)	1174.6↑	61.3↑	0.4 (-1.6, 1.7)	-1.3	5
miR-15b#	9.9 (2.6, 11.4)	6.2 (5.6, 8.4)	4.0 (2.0, 4.4)	13.3[b]	58.5[b]	-2.6 (-4.0, -1.8)	6.1[b]	11/11
miR-335#	6.1 (5.6, 8.3)	6.5 (5.2, 8.1)	4.2 (3.9, 5.1)	-1.4[b]	3.6[b]	-1.9 (-3.9, -1.0)	3.8[b]	12

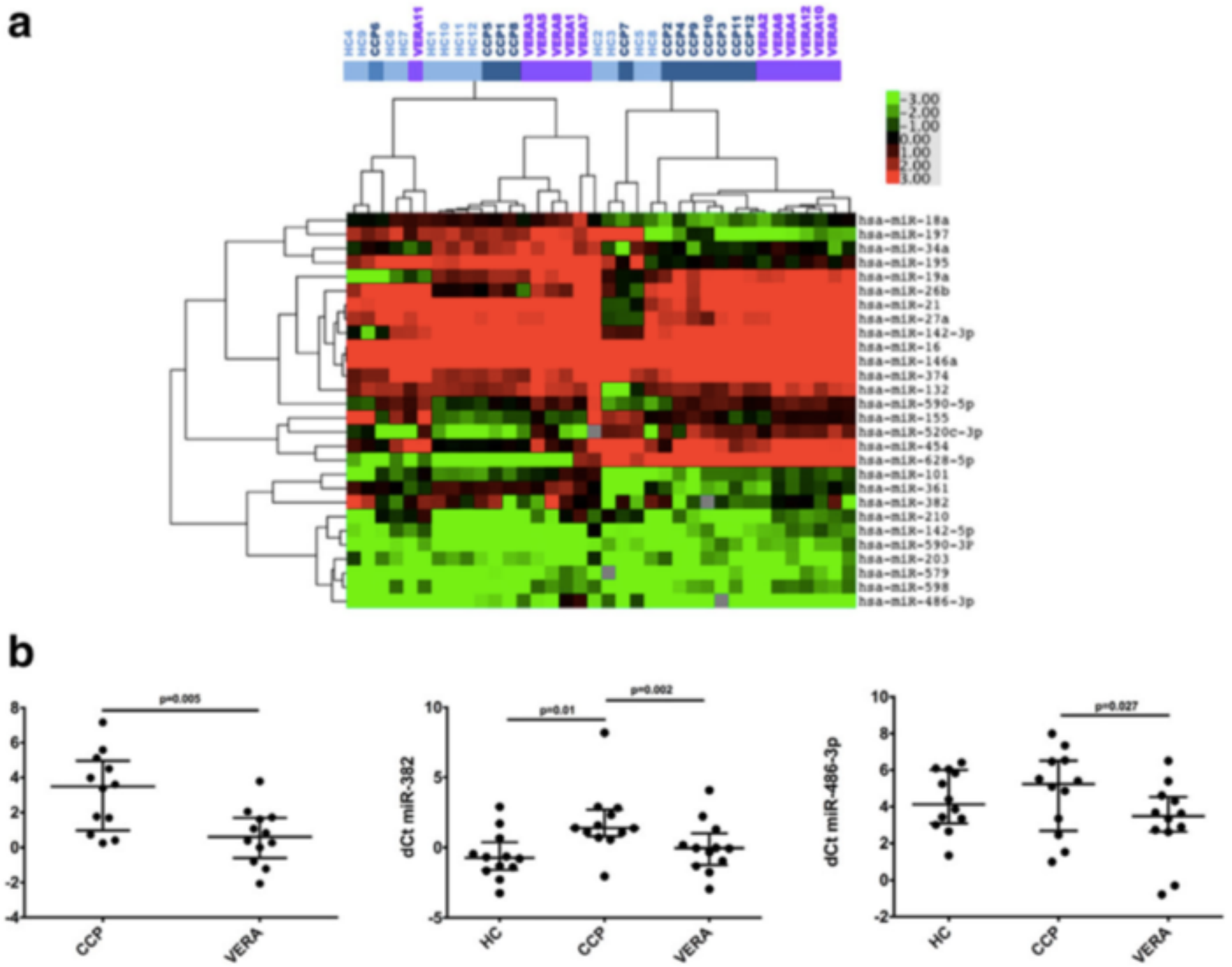

Fig. 1 Candidate serum miRNA expression profiling. **a** MicroRNA heatmaps were generated using hierarchical clustering (Gene Cluster 3.0 and Java TreeView). Green indicates low expression; red indicates high expART ression levels. **b** Comparison of expression levels of miR-22, miR-382 and miR-486-3p in matched samples from anti-cyclic citrullinated peptide (CCP) + status to very early rheumatoid arthritis (VERA) (medians, 1st to 3rd quartiles). dCt, delta threshold cycle; HC, healthy controls. Of note, miR-22 was excluded from the healthy control cohort as it was not expressed in all 12 samples

Table 2 Summary: within patient change for CCP+ status to VERA (progression) in both phases and CCP+ status to non-progression within patient change and vs. progressors from the validation phase

	CCP+ to VERA	CCP+ to no progression	CCP+ non progressor	CCP+ progressor	Progressors vs. non progressors			
	Median FC (IQR 1st, 3rd)	Median FC (IQR 1st, 3rd)	B/L median dCt (IQR)	B/L median dCt (IQR)	FD between medians	Area under ROC curve (90% CI)	Sens	Spec
Pilot phase								
miR-22	4.3 (2.8, 12.1)	-	-	-	-		-	-
miR-382	4.1 (1.7, 6.9)	-	-	-				
miR-486-3p	4.1 (0.9, 8.6)	-	-	-	-		-	-
Validation Phase								
miR-22	2.5 (-2.2, 15.3)	3.4 (2.3, 12.6)	7.4 (4.1, 8.2)	3.1 (1.8, 7.3)	19.7	0.68 (0.48, 0.82)	63%	100%
miR-382	1.2 (-2.1, 2.7)	2.4 (1.0, 2.6)	1.1 (0.0, 1.8)	-0.2 (-0.5, 1.9)	2.5	0.57 (0.40, 0.75)	75%	58%
miR-486-3p	2.2 (-2.5, 6.0)	1.0 (-1.4, 3.0)	3.4 (1.7, 3.9)	3.9 (2.6, 5.0)	-1.4	0.55 (0.36, 0.72)	50%	75%

CCP anti-cyclic citrullinated peptide, *VERA* very early rheumatoid arthritis, *miRNA* microRNA, *FC* fold change, *FD* fold difference, *B/L* baseline (sample), *IQR* interquartile range, *ROC* receiver operating characteristic, *Sens* sensitivity, *Spec* specificity

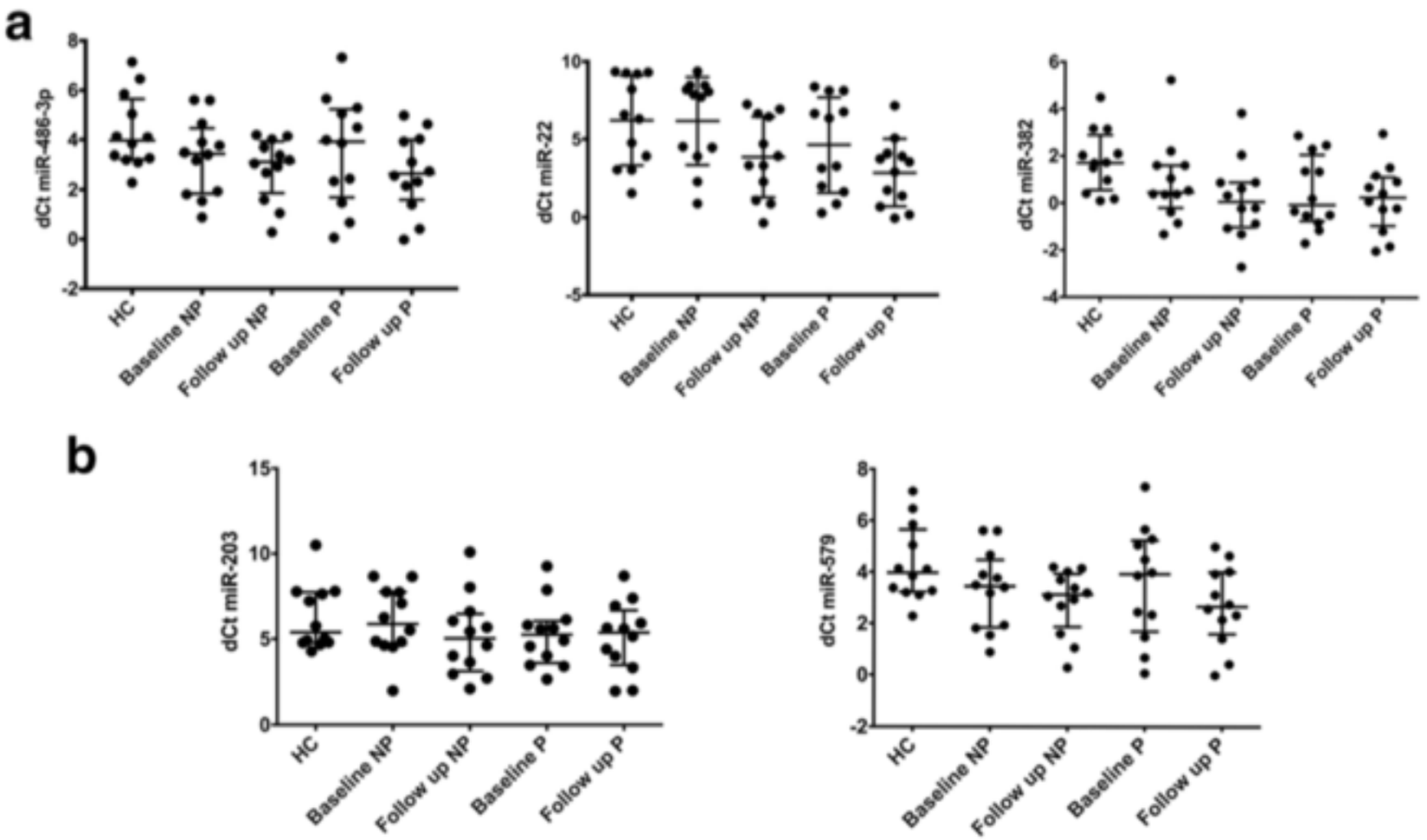

Fig. 2 Validation-phase serum expression levels of candidate miRNAs. Baseline and follow-up relative expression in the progressor (P) and non-progressor (NP) cohorts of miR-486-3p, miR-22 and miR-382 (**a**) and miR-203 and miR-579 (**b**). HC, healthy controls; dCt, delta cycle threshold

Pathway analysis and networking of miRNA target genes

Pathway prediction for the miRNAs of interest was performed, using a bioinformatics approach MetaCore™, to further elucidate functional processes associated with selected miRNAs and their targets. The expanded networks generated for miRNAs of interest represent predicted targets (Additional files 9, 10 and 11). Canonical interaction between the transcription factor p53 and miR-22 is highlighted; p53 plays a central role in a number of cellular functions, and is overexpressed in RA synovial tissue, and also activates miR-22 by binding to its promoter region [14, 15]. The predicted network shows that miR-486-3p has an inhibitory effect on bone morphogenetic protein 1 (BMP-1), indicative of miRNA function. MiR-382 negatively regulates the phosphatase and tensin homolog (PTEN), which is upstream of the AKT/mTOR signalling pathway.

Discussion

This first study of miRNAs in individuals at risk of RA has identified new miRNAs of interest, which may be associated with RA initiation and progression from systemic autoimmunity to disease and may also have a predictive role in the progression from "at-risk" status to RA.

Current literature reports clinical, serological, imaging and biological markers either associated with or potentially predictive of progression from systemic autoimmunity to RA, such as ACPA, RF, and shared epitope (SE) fine mapping [16, 17]. Other biomarkers that have been explored comprise synovial tissue and histology studies, gene expression analyses and sensitive imaging [18, 19]. MiRNA studies to date have mainly focused on

We identified potential roles of miR-22 and -382 and confirmed the importance of miR-486-3p. Despite these miRNAs in the validation phase not meeting our stringent criteria; miR-486-3p had an FC > 2, which is of biological significance particularly since there was stable expression in the non-progressors. The validation of miR-22 upregulation in VERA and CCP states compared to health potentially implicates a role in the development of inflammatory disease. Baseline miR-22 was strongly upregulated in progressors compared to non-progressors (comparator group) and thus has potential clinical utility for identifying those that may be at greatest risk. However, the ROC analysis did not reflect this, highlighting the need for further evaluation with a larger patient cohort. Interestingly it has also been identified as a predictor of response to tumour necrosis factor-inhibitor therapy [20, 21]. The higher-than-expected FC of miR-22 and miR-382 within the non-progressors may reflect an association between this miRNA with ongoing autoimmunity, which we anticipate, with further follow up in a proportion of the non-progressors, may manifest as progression to VERA. Continued evaluation of this cohort will allow us to address this.

Conclusions

In summary, we report the first study that has identified in particular three miRNAs associated with autoimmunity (at-risk RA) and the progression to RA, using a unique matched serum sample and comprehensive miRNA array approach. Given the associations between clinical markers and potential predictive ability, validation of the signature miRNAs as a next step may offer the opportunity to improve current models [5] (including evaluation in CCP+, ultrasound positive cohort), and supports investigation into the biological functions of the candidate miRNAs through future network and functional analyses.

thank you